Differentiation-Associated Localization of nPKCη, a Ca^{++}-Independent Protein Kinase C, in Normal Human Skin and Skin Diseases

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The expression of nPKCη, a Ca^{++}-independent isoform of protein kinase C in normal human skin, and skin from patients with psoriasis, squamous cell carcinoma, basal cell epithelioma, nevus pigmentosus, and seborrheic keratosis, were examined by immunohistochemical staining using a polyclonal antibody raised against a synthetic peptide at a diverse region of the nPKCη molecule. In normal epidermis, the strongest staining was observed in the uppermost granular layer with no staining of the spinous or basal layers. The inner layer of the intra-epidermal eccrine duct was also strongly stained. Weak staining was observed in several layers of the outer root sheath of the follicular infundibulum. No staining was detected in the inner root sheath of the hair follicles, hair matrix, sebaceous gland, eccrine gland, intradermal eccrine duct, arrectores pilorum, melanocytes, Langerhans cells, fibroblasts, or blood vessels. In psoriatic skin, stained keratinocytes were distributed in the suprabasal layers with the most being observed in the uppermost layer and the least in layers closed to the basal layer. In squamous cell carcinoma, weak staining was observed in the keratotic cells around horny pearls. In the basal cell epithelioma and nevus pigmentosus, the cells were not stained, whereas in seborrheic keratosis, cells that stained were located in the granular layer. We conclude from the evidence presented above that nPKCη is expressed in close association with epidermal differentiation in normal skin and skin diseases. Key words: nPKCη/differentiation-associated localization/normal epidermis. *J Invest Dermatol 101:858–863, 1993*

Transduction of extracellular signals across the cell surface involves agonist-induced hydrolytic catalysis of phosphatidylinositol-4,5-diphosphate by phosphatidylinositol-specific phospholipase C. This reaction produces two intracellular second messengers, 1,2-diacylglycerol (DAG) and inositol-1,4,5-triphosphate. The former, DAG, activates protein kinase C (PKC) whereas the latter releases calcium from intracellular stores. By implication, therefore, PKC is regarded as a key enzyme in transmembrane-signaling pathways in cells [1]. In epidermal keratinocytes, several hormones and peptides, such as bradykinin, platelet-activating factor, thrombin, and substance P, were reported to activate PKC [2–6].

PKC exists as a family of isoforms with closely related structures and enzymologic characteristics. Ten members of the PKC family have been identified to date. They are classified into three major groups: Ca^{++}-dependent conventional PKCs (cPKCα, -βI, -βII, and -γ), Ca^{++}-independent novel PKCs (nPKCδ, -ϵ, -η, and -θ), and DAG or phorbol ester-independent atypical PKC (aPKCζ and -λ) [7]. Conventional PKC isoforms contain three conserved domains, i.e., the C1 domain with two cysteine-rich zinc finger-like motives, the C2 domain determining Ca^{++}-sensitivity, and the catalytic C3 domain. They are enzymatically characterized by the requirements of Ca^{++}, phosphatidylserine, and DAG. The second group of PKC isoforms, nPKCs, lacks the C2 domain and does not show a calcium requirement [8–11]. The aPKC isoforms have only one cysteine-rich repeat in the C1 domain and are independent of Ca^{++} or DAG. The presence of multiple isoforms and their tissue-specific distributions suggest that each performs a distinct role in the growth, differentiation, and functioning of cells [1,8,11–13].

We have cloned nPKCη, a new family member of PKC, from a cDNA library of mouse skin [8]. A human version of nPKCη, termed PKC-L, was reported by Bacher *et al* [14]. Northern blot analysis has shown that the mRNA of nPKCη is highly expressed in skin and lung but only slightly in brain [8]. This unique tissue distribution prompted us to examine the possibility that nPKCη is a major PKC isoform in most epithelial tissues. Indeed, we found that nPKCη is expressed predominantly in the epithelia of the skin, digestive, and respiratory tracts in association with differentiation of epithelial cells [13].

In this communication, we describe the differentiation-associated localization of nPKCη in normal human skin and that from various skin diseases such as psoriasis, squamous cell carcinoma, basal cell epithelioma, nevus pigmentosus, and seborrheic keratosis.

MATERIALS AND METHODS

Human Skin Samples Biopsy samples of normal skin were obtained from the lower extremities of two women (22 and 62 years old) and one man (32 years old), and the nape of a second man (54 years old). Biopsy samples of the following skin conditions were obtained also: psoriatic lesions from six patients of psoriasis vulgaris (two women, 38 and 75 years old, from the arms; four men, 46, 41, 53, and 71 years old, one sample from the chest, and three from the abdominal wall, respectively); squamous cell carcinoma (one woman, 91 years old, from the face; one male, 56 years old, from a dorsal site of the hand); basal cell epithelioma (one man, 65 years old, from the arm);

Manuscript received February 19, 1993; accepted for publication June 30, 1993.

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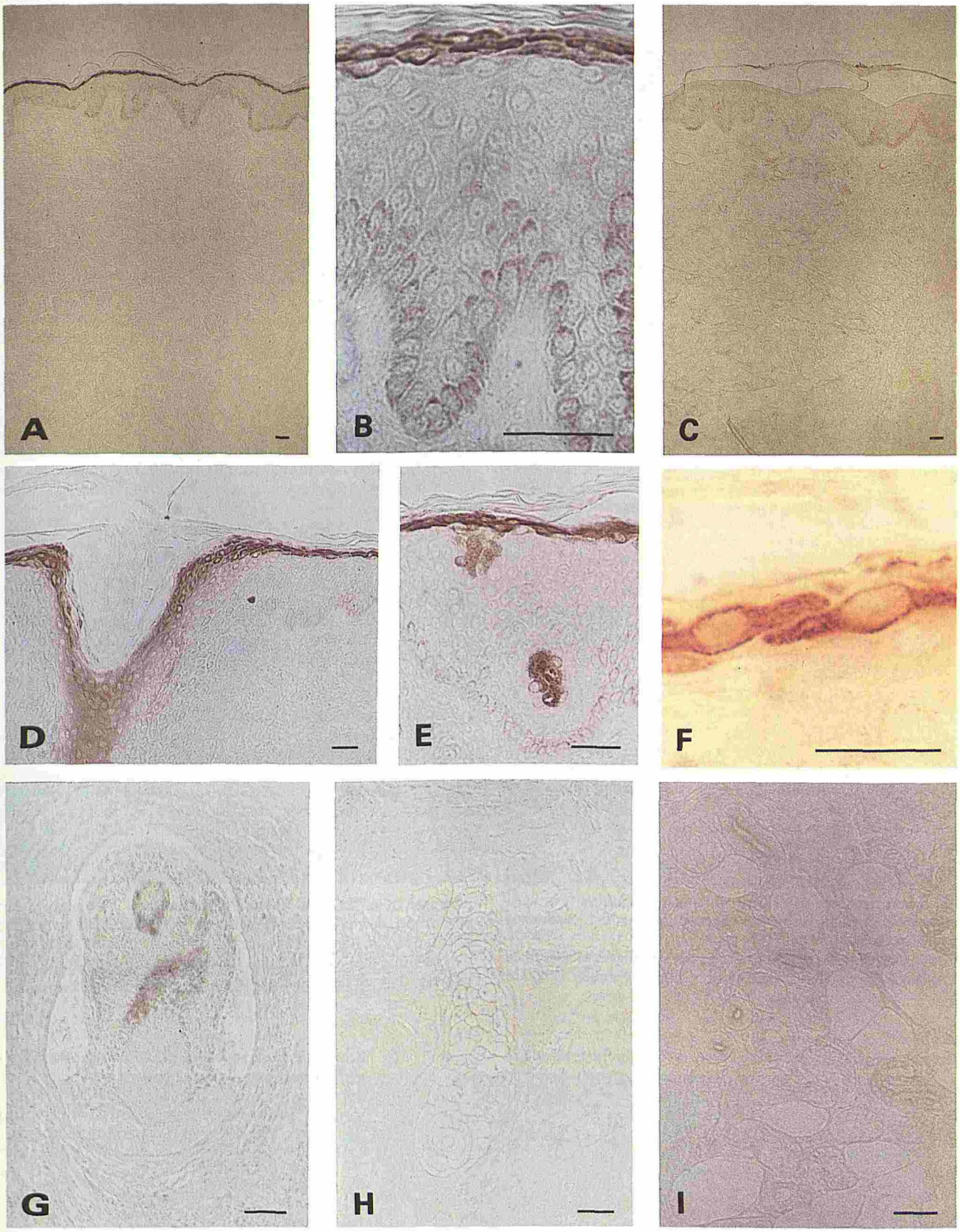

Figure 1. Immunohistochemical demonstration of nPKCη in normal human skin. *(A)* Epidermis and dermis. Note that nPKCη is located exclusively in uppermost granular layer. *(B)* High magnification of granular layer. *C)* Epidermis and dermis in which the staining is eliminated by the addition of peptide antigen. *D)* Upper portion of hair follicle in which several outer layers of the follicular infundibulum are stained. *E)* Epidermis with intraepidermal eccrine duct, which is also strongly stained. The dark staining of the basal layer is due to melanin pigment. *F)* Staining of cells in granular layer. Note that staining is strong in the cytoplasmic region devoid of nuclear space. *G–I)* Hair bulb *(G)*, sebaceous gland *(H)*, and eccrine gland *(I)* are not stained by the antibody. *Bars,* 40 μm *(A–E, G–I)* and 20 μm *(F)*.

nevus pigmentosus (one woman, 41 years old; one man, 65 years old; both samples from the face), and seborrheic keratosis (one woman, 62 years old, from the abdominal wall). The patients with psoriasis had not received treatment for the condition for at least 2 weeks before the biopsies were done, and none of the other patients had received any treatment. Biopsy materials were fixed with Bouin's solution, dehydrated, and embedded in paraffin.

Preparation of Antiserum Against nPKCη A polyclonal antibody was raised against a synthetic peptide with the sequence QTSTK QKTNKPTYNEEFC, which corresponds to the N-terminal D1 diverse region (56–73) of human and mouse nPKCη [13]. This sequence shares homology (61%, 11/18) with only one of the PKC isoforms, namely nPKCε. The antibody was purified by chromatography and its specificity was confirmed by immunoblotting; it reacted with an 82-kilodalton (Da) protein produced by COS1 cells overexpressing nPKCη, but not with the equivalent protein produced by cells expressing nPKCε.

Immunohistochemical Staining Paraffin-embedded tissues were cut into 4-μm sections and hydrated. The sections were pretreated with 3% hydrogen peroxide for 10 min at 4°C and washed with phosphate-buffered saline (PBS). They were then incubated with 10% normal horse serum at room temperature for 20 min, followed by overnight treatment with the antibody against nPKCη (diluted 1:5000 in PBS containing bovine serum albumin). After washing with PBS, the sections were reacted with biotinated F[ab″] fragment of affinity-purified porcine anti-rabbit immunoglobulin (Dakopatts A/S, Denmark) for 30 min at room temperature. Then, the specimens were incubated with peroxide-conjugated streptavidin (Dakopatts A/S, Denmark) for 30 min at room temperature, washed with PBS, and reacted with 0.1% diaminobenzidine. Some specimens were counterstained with Mayer's hematoxylin. Specimens were dehydrated and mounted. The specificity of the reaction was confirmed by elimination of the staining in the presence of an excess amount (1 μg/ml) of the peptide antigen.

RESULTS

Localization of nPKCη in Normal Skin In normal human skin, positive staining for nPKCη was observed in the keratinocytes comprising the granular layer of the interfollicular epidermis (Fig 1*A, B*). This staining was not observed in the basal or spinous layers. Melanocytes and dendritic Langerhans cells in the epidermis were not stained (data not shown).

In the hair follicle, weak staining was observed in several outer layers of the follicular infundibulum (Fig 1*D*). Neither inner root sheath, hair matrix, nor papilla showed positive staining (Fig 1*G*). The sebaceous glands and the arrectores pilorum, which consists of smooth muscle, did not stain (Fig 1*H*).

Strong positive staining was found in the cytoplasm of the inner cells of the intra-epidermal eccrine duct where granular cells are present (Fig 1*E*), but the intra-dermal eccrine ducts and eccrine glands were negatively stained (Fig 1*I*). Blood vessels, fibroblasts, and nerves in the dermis were devoid of staining. Adipose cells in the subcutaneous tissue were not stained (Fig 1*I*).

Staining was strong in the cytoplasmic region devoid of nuclear space (Fig 1*F*).

In all the instances described above, and in those that follow below, staining was eliminated by the addition of peptide antigen (Fig 1*C*, 2*C*), thus demonstrating a specific reaction with nPKCη.

Localization of nPKCη in Psoriasis Psoriatic involved epidermis shows regular acanthosis and parakeratosis with a depleted granular layer. Six biopsy samples of involved psoriatic skin were examined for localization of nPKCη. In contrast to normal skin, staining was not limited to the uppermost two or three layers but was distributed in whole suprabasal layers, with the uppermost layer staining the strongest and the lower layer the weakest (Fig 2*A*). Weak staining was occasionally observed in the basal layer. As in normal skin, the immunoreaction was detected only in the cytoplasm (Fig 2*B*). Uninvolved skin adjacent to the psoriatic involved skin showed a similar pattern of nPKCη expression as normal skin. Infiltrated cells in the upper dermis and epidermis did not stain.

Localization of nPKCη in Squamous Cell Carcinoma Two biopsy specimens of well-differentiated squamous cell carcinoma of skin were immunostained with the nPKCη antibody. Weak staining only was detected in the keratotic cells around horny pearls (Fig 3*A*). As with normal and psoriatic skin, the immunoreactive material was localized exclusively in the cytoplasm.

Absence of nPKCη in Basal Cell Epithelioma Basal cell epithelioma consists of basophilic cells with a high nucleus: cytoplasm ratio that mimic the basal cells of the epidermis. We found that cells of basal cell epithelioma did not stain with the anti-nPKCη antibody, most probably due to the absence of keratinization (Fig 3*B*). At the periphery of the tumor, acanthotic epidermis adjacent to the tumors was stained with the anti-nPKCη antibody, whereas in the center, melanin pigments were observed in dark brown.

Absence of nPKCη in Nevus Pigmentosus Two biopsy samples of intradermal nevus pigmentosus were examined for expression of nPKCη. Nevus cells were totally devoid of stain, from which we conclude that nPKCη is not expressed in cells, including melanocytes, that are derived from neural crest (Fig 3*C*).

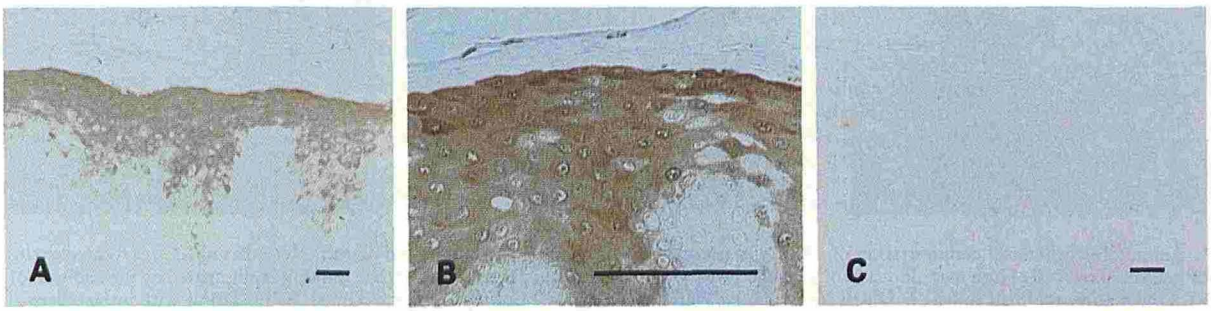

Figure 2. Immunohistochemical demonstration of nPKCη in psoriatic-involved epidermis. *A,B)* Psoriatic-involved epidermis by lower and higher magnifications. Note that stained keratinocytes are distributed in whole suprabasal layers with the uppermost layer staining the most strongly. *C)* Negative control in the presence of the antigen peptide. *Bars,* 40 μm.

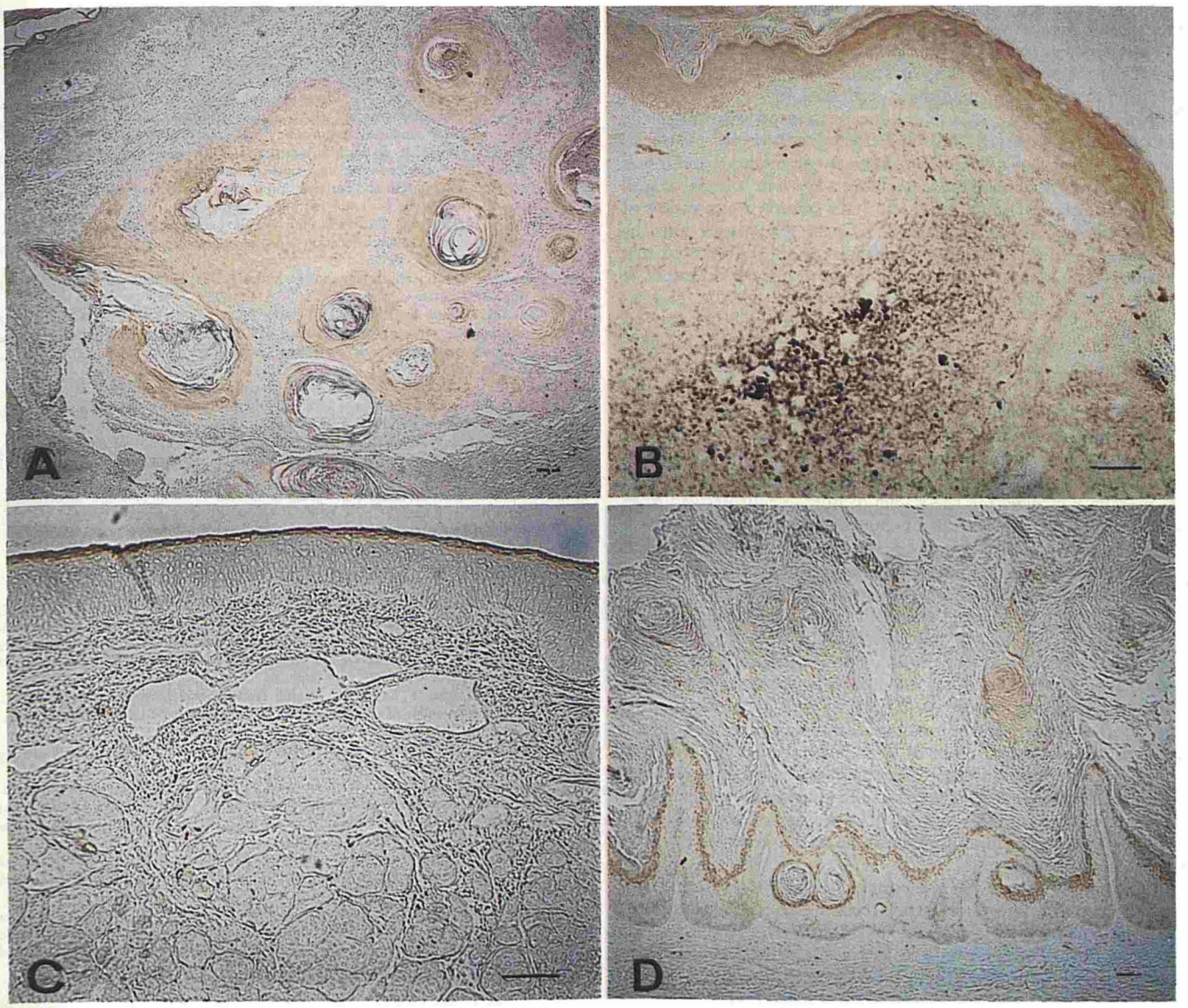

Figure 3. Immunohistochemical demonstration of nPKCη in squamous cell carcinoma *(A)*, basal cell epithelioma *(B)*, nevus pigmentosus *(C)*, and seborrheic keratosis *(D)*. In squamous cell carcinoma *(A)*, nPKCη is located only in the keratotic cells around horny pearls. The cells consisting of basal cell epithelioma *(B)* and nevus pigmentosus *(C)* are totally devoid of stain. At the periphery of basal cell epithelioma, acanthotic epidermis adjacent to the tumors was stained with the anti-nPKCη antibody, whereas in the center, melanin pigments were observed in dark brown *(B)*. Granular layer is stained in the adjacent epidermis to the tumors *(C)* and in seborrheic keratosis *(D)*. *Bars,* 80 μm.

Localization of nPKCη in Seborrheic Keratosis One biopsy specimen of the hyperkeratotic type of seborrheic keratosis was immunostained with the nPKCη antibody. The specimen showed papillomatosis with hyperkeratosis and thickening of the epidermis. An immunoreaction was detected in the four to five uppermost layers of the epidermis only, where granular cells are located (Fig 3*D*).

DISCUSSION

We have demonstrated that nPKCη, an isoform of Ca^{++}-independent PKC, is expressed in keratinocytes and inner cells of the intra-epidermal eccrine duct of human skin and these are the only cells that express nPKCη in skin. We did not detect it in melanocytes, Langerhans cells, eccrine gland, sebaceous gland, or arrectores pilorum.

The expression of nPKCη is closely associated with differentiation of keratinocytes both in normal skin and in the skin diseases we investigated. In normal skin, we observed expression of nPKCη in the uppermost granular layer, outer layers of the follicular infundibulum, and the inner layer of the intra-epidermal eccrine duct. In psoriatic skin, however, we found cells expressing nPKCη in all suprabasal layers of the epidermis, in diminishing concentration from the uppermost layer to the lowest, and indicative of aberrant differentiation of keratinocytes in psoriasis. In squamous cell carcinoma, stained keratotic cells were observed around horny pearls.

The expression of nPKCη was not apparent in the basal layer where stem cells or DNA-synthesizing cells are located. In keeping with this observation, staining was not detected in basal cell epithelioma, which consists of cells that mimic basal cells. We have reported elsewhere that little or no expression of nPKCη can be de-

tected at levels of mRNA and protein in the basal layer of interfollicular epidermis, tongue, esophagus, forestomach, or crypt of intestine of mice [13]. According to the existing data, we concluded that expression of nPKCη is associated with differentiation of epithelial cells rather than their proliferation.

There are several reports of the localization of isoforms of the PKC family in skin [15–18]. Leibersperger *et al* [16] examined tissue distribution of nPKCδ by immunologic methods and found that it was expressed in all epidermal layers and hair follicles as well as in papilloma and carcinoma. We have observed expression of cPKCα and nPKCδ along with nPKCη in the skin of mice by Northern blotting [13]. Recently Dlugosz *et al* [18] reported that keratinocytes in culture express mRNA encoding cPKCα, nPKCδ, nPKCϵ, aPKCζ, and nPKCη, but not cPKCβ or cPKCγ. The expression of cPKCβ in dendritic cells in mouse epidermis was reported by Koyama *et al* [19], which is consistent with the previous observation that immunologically competent cells express this isoform [2]. These PKC isoforms may play specific roles in the signal transduction of growth and differentiation of keratinocytes.

We demonstrated the presence of nPKCη in the cytoplasm of keratinocytes devoid of *nuclear* spaces. However, Greif *et al* [20] reported the finding that PKC-L, the human version of nPKCη, was present exclusively in the nuclei of human cells derived from keratinocytes and their malignant counterparts. However, we have not been able to confirm their observation by the use of the same cell line they used and the antibody we used in the present study (unpublished data). The reason for this is not known.

Keratohyalin granules and cornified envelopes containing loricrin, filaggrin, involucrin, and cystatin-alpha (keratolinin) are formed in the granular layer [21–26]. The insoluble cornified envelope is formed by the cross-linking of involucrin, loricrin, and cystatin-alpha by transglutaminase [27,28]. It is of particular interest that patterns of expression of involucrin and transglutaminase in the epidermis are similar to those of nPKCη in normal and psoriatic skin. By immunohistochemical staining of normal skin, involucrin and transglutaminase were detected in the granular layer and uppermost one or two layers of the spinous layer. In psoriatic epidermis, however, involucrin and transglutaminase are also found in the deep spinous layer [29,30].

PKC could be involved in epidermal differentiation by activating certain genes and phosphorylating certain enzymes. Transglutaminase is known to be induced by 12-O-tetradecanoylphorbol 13-acetate (TPA), which is a direct activator of PKC [31,32]. Involucrin may also be induced by TPA because its promoter region contains a TPA-responsive element [33,34]. Chakravarty *et al* [35] reported that TPA treatment induced phosphorylation of transglutaminase of keratinocytes, suggesting that transglutaminase is a substrate of PKC. Furthermore, cystatin-alpha is also a substrate for PKC and its phosphorylated form is contained in keratohyalin granules of granular cells [36]. Considering the localization and modification by phosphorylation of these keratinization-related molecules, nPKCη is suggested to play a key role in epidermal differentiation, and is thereby involved in skin diseases that involve disorder of epidermal differentiation.

This work was supported in part by a Grant-in-Aid for Scientific Research (Grant No. 02670469) (HK) and by a Grant-in-Aid for Special Project Research, Cancer-Bioscience (SOh, TK) from the Ministry of Education, Science and Culture of Japan.

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